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Title

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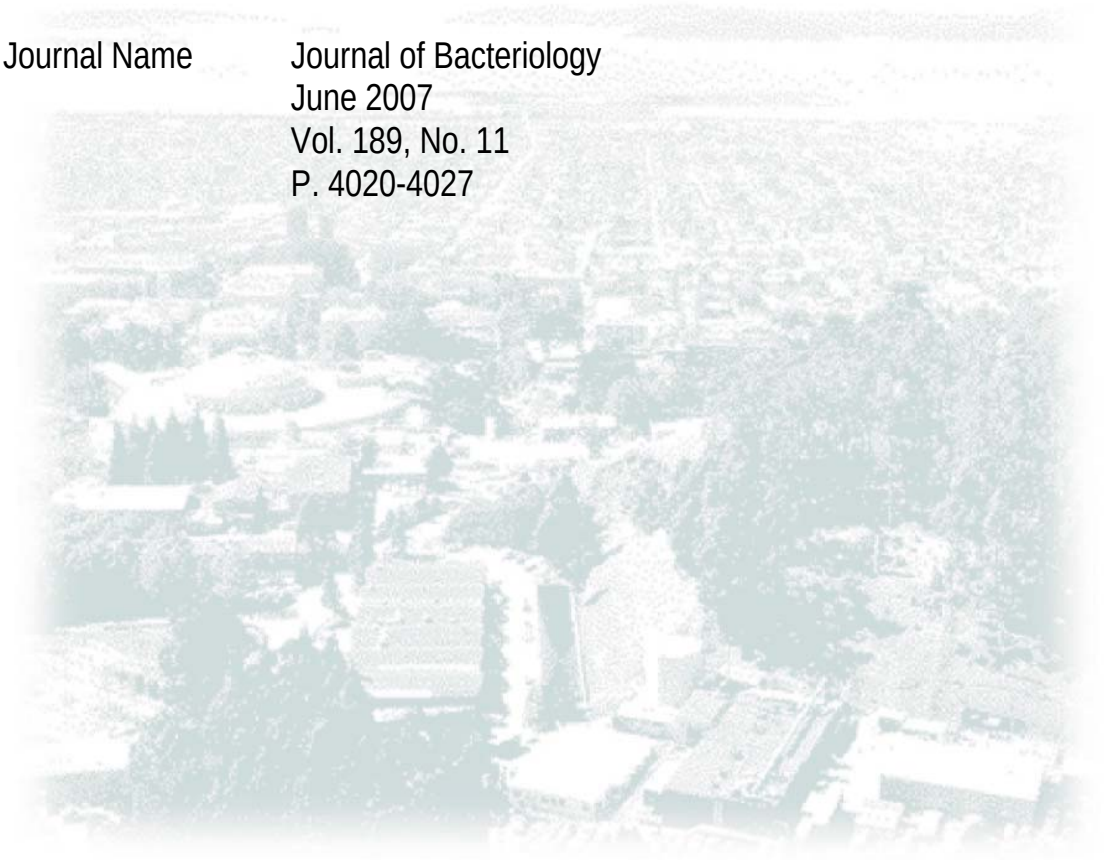

The genome of *Methylobacillus flagellatus*, the molecular basis for obligate methylotrophy, and the polyphyletic origin of methylotrophy

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Along with methane, methanol and methylated amines represent important biogenic atmospheric constituents, thus not only methanotrophs, but also non-methanotrophic methylotrophs play a significant role in global carbon cycling. The complete genome of a model obligate methanol and methylamine utilizer, *Methylobacillus flagellatus* (strain KT) was sequenced. The genome is represented by a single circular chromosome of approximately 3 Mb pairs, potentially encoding a total of 2,766 proteins. Based on genome analysis as well as the results from previous genetic and mutational analyses, methylotrophy is enabled by methanol- and methylamine dehydrogenases, the tetrahydromethanopterin-linked formaldehyde oxidation pathway, the assimilatory and dissimilatory branches of the ribulose monophosphate cycle, and by formate dehydrogenases. Some of the methylotrophy genes are present in more than one (identical or non-identical) copy. The obligate dependence on single carbon compounds appears to be due to the incomplete tricarboxylic acid cycle, as no genes potentially encoding alpha ketoglutarate, malate or succinate dehydrogenases are identifiable. The genome of *M. flagellatus* was compared, in terms of methylotrophy functions, to the previously sequenced genomes of three methylotrophs: *Methylobacterium extorquens* (Alphaproteobacterium, 7 Mbp), *Methylibium petroleophilum* (Betaproteobacterium, 4 Mbp), and *Methylococcus capsulatus* (Gammaproteobacterium, 3.3 Mbp). Strikingly, metabolically and/or phylogenetically, methylotrophy functions in *M. flagellatus* were more similar to the ones in *M. capsulatus* and *M. extorquens* than to the ones in the more closely related *M. petroleophilum*, providing the first genomic evidence for the polyphyletic origin of methylotrophy in Betaproteobacteria.

INTRODUCTION

Methylotrophy is a metabolic capacity to grow on compounds containing no C-C bonds, such as methane, methanol, methylated mines *etc.* (1, 32). While the role of methanotrophs in reduction of global emissions of methane has been well recognized (25), less attention has been paid to non-methanotrophic methylotrophs as participants of the global carbon cycle. However, recent models estimate methanol emissions into the atmosphere at 82-273 Tg y^{-1} (living plants being the major source; 16, 20), putting them on the scale similar to the scale of methane emissions (approx. 600 Tg y^{-1}; 29) and pointing toward the global role of non-methanotrophic methanol utilizers. While no global modeling has been attempted for methylated amines production, they are known to be abundant in marine and freshwater environments and represent the dynamic constituents of not only carbon, but also nitrogen global cycles (36). So far only non-methanotrophic methylotrophs have been implicated in utilizing methylated amines (1, 32).

Methylobacillus flagellatus strain KT utilizes methanol and methylated amines as single sources of carbon and energy and is classified as an obligate methylotroph (19). The strain has been isolated in early eighties from a metropolitan sever system (19) and selected as a prospective industrial strain, due to its high growth rates on methanol, high tolerance to methanol and also formaldehyde, high biomass yield and a high coefficient of conversion of methanol into biomass (2, 3, 6, 19). Derivatives of the strain have been successfully generated aimed at commercial production of value added compounds (5, 17, 34). In addition to its commercial potential, the strain became one of the most prominent models in studying biochemistry of methylotrophy as it presents a facile

genetic system, with a variety of tools for manipulation such as suicide vectors for site-directed mutagenesis, expression vectors, promoter probe vectors *etc.* (7, 13, 15, 27). Thus, the genome-based analysis of methylotrophy in this organism is complemented by a body of previous genetic and biochemical data. Based on 16S rRNA sequence, *M. flagellatus* belongs to Betaproteobacteria and is most closely related to other members of *Methylophilaceae* (19). The genomic sequence of *M. flagellatus* reported here presents an excellent study case for comparative analysis of molecular basis of methylotrophy in alpha-, beta- and gammaproteobacteria.

MATERIALS AND METHODS

Methylobacillus flagellatus strain KT (ATCC 51484) was from the laboratory collection. For the isolation of genomic DNA, cultures were grown in 100 ml of minimal medium (21) supplemented with 2% (W/V) methanol. Genomic DNA was isolated from late-exponential-phase cultures in accordance with the recommendations by the Department of Energy's Joint Genome Institute (DOE-JGI; Walnut Creek, CA). The genome was sequenced using the whole-genome shotgun method (13) and assembled using standard tools as described on the JGI website (http://www.jgi.doe.gov/). Gaps remaining in the assembled sequence were closed by primer walking or by sequencing specifically amplified PCR fragments. Sequence was finished and polished at the ??? facility and computationally annotated at the ??? facility. Automated functional assignments were curated manually, as appropriate. The sequence of the complete *M. flagellatus* strain KT genome is available under GenBank accession number NC 007947

and also at the JGI web site (http://genome.jgi-psf.org/finished_microbes/metfl/metfl.home.html).

RESULTS

General genome features and basic functions

The genome consists of a single circular chromosome of 2,971,517 base pairs (55.7 % GC content) of which 143,032 base pairs represent a direct identical repeat (see below). A total of 2,766 coding regions are recognized in the genome, of which 144 are identical doubles as these are parts of the extended repeat. Of the total translatable open reading frames, 233 are unique to *M. flagellatus*, 2,520 have top BLAST hits with bacterial genes, 10 have top hits with archaeal genes, and 3 have top hits with eukaryotic genes. Based on protein identity scores, the closest relatives of *M. flagellatus* whose complete genome sequences are available are the Betaproteobacteria *Thiobacillus denitrificans* (492 of a total of 1681 betaproteobacterial top hits) and *Dechloromonas aromatica* (299 top hits). Of 568 gammaproteobacterial top hits, 108 are with the proteins translated from the chromosome of *Methylococcus capsulatus*, an obligate methane utilizer whose genome has been recently sequenced (44). Some of these are the well-characterized methylotrophy genes (see below), while most of the remaining genes are hypothetical genes, and some of these may be involved in methylotrophy as well. The chromosome contains two ribosomal (16S-23S-5S) operons and all the genes encoding ribosomal proteins. There are a total of 46 tRNA genes corresponding to 38 tRNA acceptors for recognizing all 20 amino acids. Only one complete (Tn3 type) transposase gene is present in the genome (Mfla1495), and a partial gene is present nearby

(Mfla1488), the two surrounding a group of genes predicted to be involved in arsenate resistance. A number of phage-related genes were identified in the genome, and most of these appear to have homologs in related Betaproteobacteria, with a few exceptions that are unique to *M. flagellatus* (see below). The genome of *M. flagellatus*, like many microbial genomes (33), contains a CRISPR repeat (93 identical sequences of 32 nucleotides interspaced by non-identical sequences of 33 to 39 nucleotides; nucleotides 632591-638803) that is non-homologous to any known CRISPR repeats. The repeat is preceded by six genes encoding CRISPR-associated proteins (Mfla 601-607).

Standard sets of genes are present for DNA replication, transcription and translation, and complete pathways are apparently present to synthesize all the amino acids, nucleotides, and a few vitamins. Few secondary metabolite synthesis pathways are encoded in the genome, for example, a pathway for terpenoid precursor (geranylgeranyl) biosynthesis. A single large gene cluster (Mfla1940-1987) is responsible for encoding the flagellum functions. A complete set of genes for NADH quinone oxidoreductase (complex I) were identified (Mfla2048-2061), all in one cluster, as well as the genes for cytochrome c oxidase (complex IV, Mfla1292-1295). An array of genes were predicted to be involved in regulatory functions, including numerous *lysR*, *tetR* and *luxR* homologs, as well as other classes of regulators. However, little knowledge exists on the regulation of either methylotrophy or general metabolism function in *M. flagellatus*. Thus the specific functions of the predicted regulators will need to be tested via mutation and/or expression analyses in the future. A large number of genes encoding putative transporters were identified in the genome. The 31 TonB-dependent siderophore receptor gene homologs are likely involved in iron uptake. Other transporters are predicted to be involved in

transport of other metals or in nitrate, ammonium, or sulfate metabolism, as well as in biopolimer transport. Type I, II and IV secretion systems are also encoded. A cluster of genes encoding parts of a phosphotransferase-type sugar transport system was identified, similar to the clusters previously characterized in *Nitrosomonas europea* (24) and *Nitrosococcus oceany* (31). However, the function of these genes remains enigmatic (24).

Methylotrophy

Primary oxidation of methanol and methylamine

M. flagellatus exhibits very high growth rates on methanol or methylamine (up to 0.73 h^{-1}; 2, 3, 6) and possesses high activities of methanol and methylamine dehydrogenases (MDH and MADH, respectively; 6, 30). The genome analysis revealed the presence of a gene cluster encoding MDH and accessory proteins (*mxaFJGIRSACKLD*, Mfla2034-2044) similar to the clusters characterized in other methylotrophs (8, 22, 44). In *Methylobacterium extorquens* this cluster contains two additional genes, *mxaEH* whose functions remain unknown (8), but no homologs were found in the genome of *M. flagellatus*. In addition to the bona fide MDH gene cluster, four additional gene clusters were identified in the genome encoding homologs of the large subunit of MDH (*mxaF*; Mfla344, 1451, 1717 and 2314), three of them linked to genes encoding cytochromes *c* (Mfla342, 1450, and 2312, respectively), one of the clusters also containing a homolog of *mxaJ* (Mfla2313), but none containing homologs of *mxaI* that encodes the small subunit of MDH (8). Two additional clusters were identified encoding functions essential for synthesis of active MDH (*mxaRSACKL*, Mfla687-692 and Mfla1895-1900), but these were not linked to any genes potentially encoding

pyrolloquinoline quinine (PQQ)-linked dehydrogenases. An additional cluster encoding homologs of *MxaED* (Mfla2124 and 2125) was also identified. Homologs of the genes for MDH subunits as well as other MDH functions are often found in the genomes of both methylotrophs and non-methylotrophs (9, 12, 22, 39, 44). However, mutant analysis suggests that these homologs are not involved in methanol oxidation, and a MDH proper, composed of the small and the large subunits, is required (9). Genes for biosynthesis of PQQ, the cofactor of MDH, were found in two separate clusters (*pqqABCDE*, Mfla1680-1683, 18) and *pqqFG*, Mfla734-735), similar to the clusters previously characterized in other methylotrophs (8, 22, 44).

All the genes for MADH synthesis have been partially identified previously and all are present in a single cluster on the chromosome (*mauFBEDAGLMNazu*, Mfla547-556; 14, 15). No genes with high homologies to the regulatory genes involved in methanol- or methylamine oxidation functions that have been characterized in *M. extorquens* (8) or *Paracocccus denitrificans* (11, 23) are identifiable in the *M. flagtellatus* chromosome, pointing toward the existence of non-homologous regulatory systems.

Formaldehyde oxidation

Genes for two pathways for formaldehyde oxidation are present, the oxidative branch of the ribulosemonophosphate (RuMP) cycle and the tetrahydromethanopterin (H_4MPT)-linked formaldehyde oxidation pathway (Fig. 1). Based on experimental evidence, the former is the essential pathway and the latter is auxiliary (7, 27). There are two enzymes that are specific to the RuMP cycle, hexulosephosphate synthase (HPS) and hexulosephosphate isomerase (HPI; 1). Two copies of *hps* were identified (Mfla250 and

Mfla1654), one is part of a gene cluster previously identified in "*Aminomonas aminofaciens*" (an uncharacterized *Methylobacillus* species) and containing genes for histidine biosynthesis (40), while the other is part of the previously characterized large methylotrophy gene cluster containing most of the genes for the H_4MPT-linked formaldehyde oxidation pathway, the so-called "archaeal-like" gene cluster (27). Genes for HPI (Mfla1653) and transaldolase (Mfla1655) are also found in this cluster. Physical linking on the chromosome of RuMP cycle genes and the H_4MPT-linked pathway genes is so far unique to *Methylophilaceae* (27). Like HPS, the first enzyme of the RuMP cycle, Fae, the first enzyme of the H_4MPT-linked pathway (responsible for condensation of formaldehyde with H_4MPT; 43) is also encoded by two different genes. The first is part of the main "archaeal" gene cluster (Mfla1652; 27), while the second (Mfla2543) does not appear to be linked to any recognizable methylotrophy genes. The proteins translated from the two genes are 83% identical. In addition to the two *bona fide fae*, two *fae* homologs are present, previously designated as *fae2* (Mfla2524) and *fae3* (Mfla2364), and the functions of these genes remain unknown (27). Four additional "archaeal-like" genes involved in H_4MPT-linked formaldehyde oxidation pathway (*afp, orf20, orf19, orf22*, Mfla1579-1582) form a separate gene cluster (27).

The product of HPI, fructose 6-phosphate (P) is converted to glucose 6-P by phosphoglucoisomerase (1). A single *pgi* gene is identifiable in the genome (Mfla1325), not linked to any other C_1 genes. The genes encoding glucose 6-P dehydrogenase, 6-P-gluconelactonase and 6-P-gluconate dehydrogenase (GND) form a gene cluster (*zwf-gndA-pgl*; Mfla917-919 and Mfla1061-1063) in which *gndA* and *zwf* are co-transcribed while *pgl* is transcribed in the opposite orientation, and this cluster is present in two

copies, one of which is part of the extended identical repeat. An additional copy of *gnd* (*gndB,* Mfla2599) is present in the genome. This latter gene encodes an enzyme 70% identical to GND translated from the genome of *Methylococcus capsulatus* while it is only 28% identical to *gndA.* We have previously demonstrated that overexpressing *gndA* leads to an increase in GND activity linked to NAD, while the NADP-linked activity remains unaffected (7). Likely, *gndB* is responsible for the latter activity.

Formate oxidation

Genes for a formate dehydrogenase (FDH, Mfla718-722) homologous to the one encoded in the genome of *M. capsulatus* (44) were identified in the genome. In addition, a gene is present (Mfla338) homologous to the gene recently identified as responsible for a novel FDH (FDH4) in *M. extorquens* (Chistoserdova and Lidstrom, unpublished).

Formaldehyde assimilation

The assimilatory RuMP cycle branches from the dissimilatory RuMP cycle at the level of 6-P-gluconate (1). Previous enzyme evidence suggested that the 6-P-gluconate dehydratase (KDPG)/ ketodeoxy-P-gluconate aldolase/transaldolase version of the RuMP cycle must be operational (30). Indeed, genes for all the enzymes involved are identifiable in the genome (Fig. 1). A total of three genes were identified encoding pentose phosphate isomerase (Mfla129, 962, and 1106), the latter two are identical copies (as a result of the extended repeat) having 41% amino acid identity with the former. Functioning of the alternative cleavage/regeneration versions of the RuMP cycle (fructose bisphosphate aldolase/sedoheptulose bisphosphatase, fructose bisphosphate

aldolase/TA, or KDPG/sedoheptulose bisphosphatase; 1) is not supported by genome analysis, as genes for neither phosphofructokinase, no sedoheptulose bisphosphatase are identifiable.

Pyruvate is the 'end product' of the RuMP cycle in *M. flagellatus* (Fig. 1, 1). To provide necessary cell constituent precursors, reactions converting pyruvate or acetyl-CoA into phosphoenolpyruvate (PEP) and oxaloacetate (OAA) are necessary (1). We were able to identify putative genes for PEP synthase (Mfla2203) and pyruvate kinase (Mfla2244) that are likely responsible for interconverting pyruvate and PEP. We also identified a putative gene for pyruvate carboxylase (Mfla1511) that may be responsible for converting pyruvate into OAA. In addition, a gene was identified encoding a product homologous to the alpha subunit of OAA decarboxylase (Mfla1512), another enzyme capable of converting pyruvate into OAA. However, we were not able to identify genes for the beta and gamma subunits associated with this activity (10). An alternative way of synthesizing PEP would be from glyceraldehyde phosphate (GAP) as shown in Fig. 1. This proposed metabolic loop could serve to balance the levels of pyruvate, GAP and PEP in the cell.

The tricarboxylic acid cycle (TCA) deficiency as a cause of obligate methylotrophy

Some methylotrophs (classified as facultative) can grow on multicarbon substrates in addition to C_1 substrates, while others (classified as obligate) can only grow on C_1 substrates (1, 32). The causes of obligate methylotrophy remained poorly understood. The lack of a complete tricarboxylic acid cycle (TCA) has been suggested as one of the main causes of obligate methylotrophy (1). However, the recent sequencing of

an obligate methane utilizer *M. capsulatus* revealed the presence of all the genes for the TCA (44). This is also the case with chemolithoautotrophic ammonia-oxidizing bacteria (24, 31). On the contrary, analysis of the *M. flagellatus* genome revealed that genes for three enzymes of the TCA were not identifiable (encoding malate, alpha ketoglutarate and succinate dehydrogenases). Thus in the case of *M. flagellatus*, the obligate methylotrophy may be explained by the lack of the main energy-generating pathway for multicarbon substrate metabolism. Gene candidates for the reactions leading to the formation of alpha-ketoglutarate were identified (Mfla61, 1817, 2074-2076, 2139; Fig. 1), suggesting an anapleurotic function for the partial TCA. A gene encoding an alternative enzyme for converting OAA into malate, malate:quinone oxidoreductase (Mfla11) is present in the genome, likely providing a source of malate for cell biosyntheses. Genes are also present for interconverting succinate and succinyl-CoA (Mfla1888, 1889). However, we were unable to make prediction as to how succinate or succinyl-CoA may be made to be parts of central metabolism.

Polysaccharide synthesis

Methylotrophs employing the RuMP cycle for formaldehyde assimilation are known to produce large amounts of exogenous polysaccharide (41, 45, 46). The polysaccharide may be a means to balance carbon assimilation and energy generation in specific conditions, a means for detoxifying formaldehyde (38), or an agent essential for existence of these microbes in the environment (for example, function in biofilm formation; 4). This metabolic peculiarity of methylotrophs has been explored in terms of commercial production of EPS as a food additive (45, 46). A *Methylobacillus* strain

closely related to *M. flagellatus*, *Methylobacillus* sp. Strain 12S has been employed in studies aiming to define the set of genes involved in EPS synthesis and defining their specific functions (45, 46). As a result, a cluster of 21 genes has been characterized, and chemical properties of the EPS, named methanolan, studied. The latter has been found to be a heteropolymer composed of glycosyl, galactosyl, and mannosyl residues (3:1:1; 46). Interestingly, a large gene cluster was detected in the genome of *M. flagellatus* (Mfla2007-20299) that revealed significant gene syntheny with the cluster in *Methylobacillus* sp. Strain 12S (45). However, similarity between the polypeptide counterparts was very low, not exceeding 51% identity. As homologs of most of the genes involved in EPS biosynthesis in *Methylobacillus* sp. Strain 12S were present, similar chemical properties could be predicted for the EPS excreted by *M. flagellatus*. However, significant divergence in gene sequence suggests separate histories for the respective gene clusters in *M. flagellatus* and *Methylobacillus* sp. Strain 12S. An additional gene cluster predicted to be involved in EPS biosynthesis was identified in the genome (Mfla1268-1280) containing a number of genes with (distant) homologs in the former gene cluster. This gene cluster may be involved in biosynthesis of a different EPS.

As sugar phosphates are central intermediates in the metabolism of C_1 compounds by *M. flagellatus*, theoretically, precursors for EPS biosynthesis could be drawn straight from the RuMP cycle. Alternatively, sugar phosphate precursors may be synthesized *de novo*, via the reactions of gluconeogenesis, as shown in Fig. 1. There are two arguments in favor of the enzymes in question being involved in gluconeogenesis as opposed to glycolysis, the latter theoretically allowing growth on glucose and fructose: (1) the

apparent lack of sugar phosphorilation enzymes and (2) the apparent lack of a gene for phosphofructokinase.

The identical repeat as a sign of the recent evolution of the genome

A direct repeat of 143,032 base pairs was identified in the genome. The analysis of the repeated sequence and its flanking regions has shown that part of the repeat is made up by a group of genes unique to *M. flagellatus* and most probably representing a prophage (Mfla820-832 and Mfla964-976), based on predictions that some of these genes encode phage-related functions. The sequence analysis also revealed that one copy of the putative prophage interrupts a *tonB*-independent repressor gene homolog (Mfla818 and Mfla 833), while the entire sequence of the repeat interrupts a *spoU* gene homolog (Mfla 963 and Mfla1107). The structure of the repeat schematically represented in Fig. 2 points toward the possibility that the duplication event occurred as a result of phage integration. The duplication in the sequenced strain of *M. flagellatus* appears to be a recent event, based on previous mutagenesis experiments. The repeat contains a number of C_1 utilization genes, such as *zwf* and *gndA* that are essential for survival of *M. flagellatus*. Attempts of mutagenizing these genes, via site-specific insertion mutagenesis were unsuccessful as recently as 1999, consistent with the essential role of these genes (7). Thus we assume that the duplication has occurred between 1999 and 2002, when the shotgun library for this sequencing project has been constructed. (I am trying to test this by doing mutagenesis again).

The polyphyletic nature of methylotrophy as deduced from genome comparisons

M. flagellatus is the fourth methylotroph whose genomic determinants for methylotrophy are being reported. The organisms previously characterized in these terms include *M. extorquens*, an alphaproteobacterial facultative methylotroph (8), *M. capsulatus*, a gammaproteobacterial obligate methylotroph (44), and the recently described *Methylibium petroleophilum*, a betaproteobacterial facultative methylotroph (35, Hristova et al., Submitted). Methylotrophy has been characterized before in terms of functional metabolic modules, which encompass enzymes and factors involved in a single metabolic goal, such as methanol oxidation, formaldehyde oxidation or C_1 assimilation (8). The major metabolic modules involved in methylotrophy in *M. flagellatus*, as described above, include oxidation systems for methanol and methylamine, the RuMP cycle for formaldehyde oxidation that overlaps to a large degree with the assimilatory RuMP cycle, and the H_4MPT-linked formaldehyde oxidation pathway. While at least two putative formate dehydrogenases are encoded in the genome, their contribution to methylotrophy is predicted to be minor, based on the previous experiments demonstrating low levels of FDH activity during growth on C_1 compounds and the predominant role of cyclic oxidation of formaldehyde that does not involve formate as an intermediate (6, 30). In terms of primary C_1 oxidation functions, genome comparisons revealed that gene clusters encoding the methanol dehydrogenase function in *M. flagellatus* were similar to the respective gene clusters in *M. extorquens* and *M. capsulatus* (8, 44), while no major methanol oxidation cluster encoding the large and the small subunits of MDH and an associated cytochrome (MxaG) were identified in *M. petroleophilum* (Hristova et al., Submitted). The nature of the enzyme responsible for methanol oxidation in this

organism remains unknown. The *mxaF* homolog, *xoxF* that has been identified in the genome of *M. petroleophilum*, if active, would represent a different module, along with *xoxGJ*. Homologs of *xoxFJG* are also present in *M. flagellatus, M. capsulatus* and *M. extorquens*, and in the latter organism mutation analysis failed to establish a function for this module in methanol oxidation (9). The methylamine utilization gene cluster in *M. flagellatus* was found to be similar with the one in *M. extorquens*, except for the gene for amycianin, a natural electron acceptor for MADH in *M. extorquens* was missing from the *M. flagellatus* cluster. Instead, a gene for azurine, an alternative electron acceptor was present (14, 15). No genes encoding methylamine oxidation were detected in the *M. petroleophilum* or the *M. capsulatus* genomes. For formaldehyde assimilation, *M. flagellatus* employs the KDPG/TA version of the RuMP cycle, and the same module is employed by *M. capsulatus* (30, 44). In contrast, *M. petroleophilum* does not encode key function of the RuMP cycle. Instead, its genome contains a complete set of genes for the serine cycle, the formaldehyde assimilation pathway also employed by *M. extorquens* (Table 1; 8). Different gene clustering patterns and low gene similarity between the two organisms do not imply a recent transfer from an alpha-proteobacterial methylotroph into *M. petroleophilum*. The only methylotrophy module shared by all the organisms involved in comparisons, besides formate dehydrogenases that are ubiquitous, was the H_4MPT-linked C_1 transfer module. We have previously conducted comparative analyses of gene clusters encoding H_4MPT-linked C_1 transfer reactions in methylotrophs (27). These analyses have uncovered that the cluster in *M. flagellatus* was more similar, in terms of gene syntheny, to the clusters in gammaproteobacterial methanotrophs than to the clusters in two other beta-proteobacteria, *M. petroleophilum* and *Burkholderia xenovorans*.

Phylogenetic analyses further supported the finding that in terms of H_4MPT-linked C_1 transfer functions, *M. flagellatus* is more closely related to *M. capsulatus* than to betaproteobacteria of the order *Burkhorderiales* (27). These analyses suggest that *M. flagellatus* (and other *Methylophilaceae*) and *M. petroleophilum* (and other *Burkholderiales*) have acquired genes for H_4MPT-linked C_1 transfers as results of at least two independent events. Considering the lack of other overlapping methylotrophy modules in *Methylophilaceae* and *Burkholderiales*, we propose that methylotrophy as a metabolic capacity evolved at least twice in Betaproteobacteria.

DISCUSSION

We described here the findings form the genome analysis of an obligate methanol and methylamine utilizer, *M. flagellatus* strain KT that represents a large and environmentally abundant group of methylotrophs belonging to the family *Methylophilaceae* (37). In terms of methylotrophy functions, genome analysis revealed few surprises. Sets of genes encoding methylotrophy pathways previously predicted based on biochemical and genetic analyses (7, 15, 18, 30) were identified. Some of the methylotrophy genes were found in more than one, homologous or non-homologous copy. In addition, genes for enzymes converting pyruvate, the ‘end product’ of the assimilatory RuMP cycle into PEP and OAA were identified. *M. flagellatus* excretes large amounts of EPS during growth, equaling up to 20% of total biomass (41). All the genes encoding gluconeogenesis enzymes are present in the genome, and these are implicated in EPS biosynthesis. On the contrary, operation of the Embden-Meyerhof-Parnas pathway is unlikely as no gene for phosphofructokinase is identifiable in the

genome. As expected, genes for few known sugar transporters were identified in the genome. While a set of genes homologous to a fructose transport (PTS) system were identified, they are not predicted to encode a functional transporter (24, 31). However, previous experiments on stimulation of biomass yield on methanol by the addition of glucose (30), likely due to enhanced EPS production, indicate that *M. flagellatus* is able to uptake sugars, even if non-specifically. The main cause for obligate methylotrophy of *M. flagellatus* must be the incomplete TCA as the genome is lacking three enzymes essential to its operation. While the function of malate dehydrogenase may be replaced by malate:quinone oxidoreductase (28), no enzymes that would functionally replace alpha-ketoglutarate or sucinate dehydrogenases are encoded in the genome.

The *M. flagellatus* genome encodes biosynthesis of all the amino acids and nucleotides, and of the vitamins and cofactors essential for its metabolism (biotin, riboflavin, CoA, H_4F, H_4MPT). Transport systems involved in essential metal (iron, molybdenum) homeostasis are identifiable, while few transporters predicted to uptake complex organic compounds (such as amino acids) are present. Few secondary metabolite biosynthesis pathways (such as antibiotic biosynthesis) are encoded, and no known xenobiotic degradation pathways are encoded. Overall, *M. flagellatus* appears to possess a streamlined, compact genome encoding few metabolic capacities in excess of the ones devoted to the efficient growth on C_1 compounds, possibly pointing to the unique environmental function of *M. flagellatus* and likely other *Methylotrophilaceae* in consuming C_1 compounds.

The availability of the genomic sequence of *M. flagellatus* allowed comparisons with other methyltoroph genomes, in terms of methylotrophy functions. It is remarkable

that in terms of methylotrophy metabolic modules, *M flagellatus* has more in common with *M. capsulatus*, a gammaproteobacterium than with *M. petroleophilum*, a betaproteobacterium. While biochemistry of methylotrophy in *M. petroleophilum* is not nearly as well studied as in *M. flagellatus*, some of the essential methylotrophy modules are clearly missing from its genome, such as a gene cluster encoding a bona fide methanol dehydrogenase or key genes for the RuMP cycle (Hristova et al., submitted). Instead, a complete serine cycle for formaldehyde assimilation that was believed until recently to be characteristic of alphaproteobacterial methylotrophs (1, 32) is encoded in the *M. petroleophilum* genome (Hristova et al., submitted). The only methylotrophy module shared by *M. flagellatus* and *M. petroleophilum* is the H_4MPT-linked formaldehyde oxidation pathway. However, previous phylogenetic analyses argued that even in terms of this module *M. flagellatus* is more related to gammaproteobacterial methylotrophs than to *M. petroleophilum* (27). While the questions of evolution of methylotrophy as a metabolic capability are far from being answered, it is rather clear that at least in betaproteobacteria methylotrophy has evolved more than once. It is worth noting that *M. petroleophilum* is not an isolated case of an organism possessing 'non-canonical' methylotrophy metabolic modules. We have recently characterized a group of strains, classified as *Methyloversatilis universalis* of the family *Rhodocyclaceae*, which, like *M. petroleophilum*, do not appear to possess classical dehydrogenases for methanol or methylamine and utilize serine cycle for formaldehyde assimilation (26). The rather close relatedness of *Methylophilalales, Rhodocuyclales* and *Burkholderiales* suggests recent evolution for one of the two, and possibly both distinct modes of methylotrophy within betaproteobacteria.

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Table 1

Methylotrophy metabolic modules in *M. flagellatus,* compared to other methylotrophs

Methylotrophy module	*M. flagellatus*	*M. petroleophilum*	*M. capsulatus*	*M. extorquens*
Methane monooxygenase	-	-	+	-
Methanol mehydrogenase	+	-	+	+
Mehylamine mehydrogenase	+	-	-	+
H_4MPT-linked C_1 transfer	+	+	+	+
Ribulosemono-phopshate cycle	+	-	+	-
Serine cycle	-	+	+	+
CBB cycle	-	+	+	-

FIGURE LEGENDS

Figure 1. Central metabolism of *M. flagellatus* as deduced from the genome sequence and prior genetic/physiological studies. Grey boxes indicate specific methylotrophy metabolic modules. Enzymes responsible for specific reactions are represented by the numbers (Mfla) of open reading frames as translated from the genome sequence (Genbank accession NC 007947). CH_3OH, methanol; CH_3NH_2, methylamine; CH_2O, formaldehyde; H_4MPT, tetrahydromethanopterin; H6P, hexulose 6-phosphate; F6F, fructose 6-phosphate; G6P, glucose 6-phosphate; 6PGL, 6-phosphogluconolactone; 6PG, 6-phosphogluconate; KDPG, ketodeoxy 6-phosphogluconate; Ru5P, ribulose 5-phosphate; Xu5P, xilulose 5-phosphate; E4P, eritrose 4-phopshate; S7P, sedoheptulose 7-phopshate; GAP, glyceraldehyde phosphate; DHAP, dihydroxyacetone phosphate; F16PP, fructose 1,6-bisphosphate; G1P, glucose 1-phosphate; 1,3DPG, 1,3 diphosphoglycerate; 3PG, 3-phosphoglycerate; 2PG, 2-phosphoglycerate; PEP, phosphoenolpyruvate; OAA, oxaloacetate; AcCoA, acetyl-CoA; α-KG, alphaketoglutarate; EPS, exopolysaccharide.

Figure 2. Schematic representation of the chromosomal region containing a direct identical repeat. 3’ and 5’ indicate 3’ and 5’ partial genes, respectively.

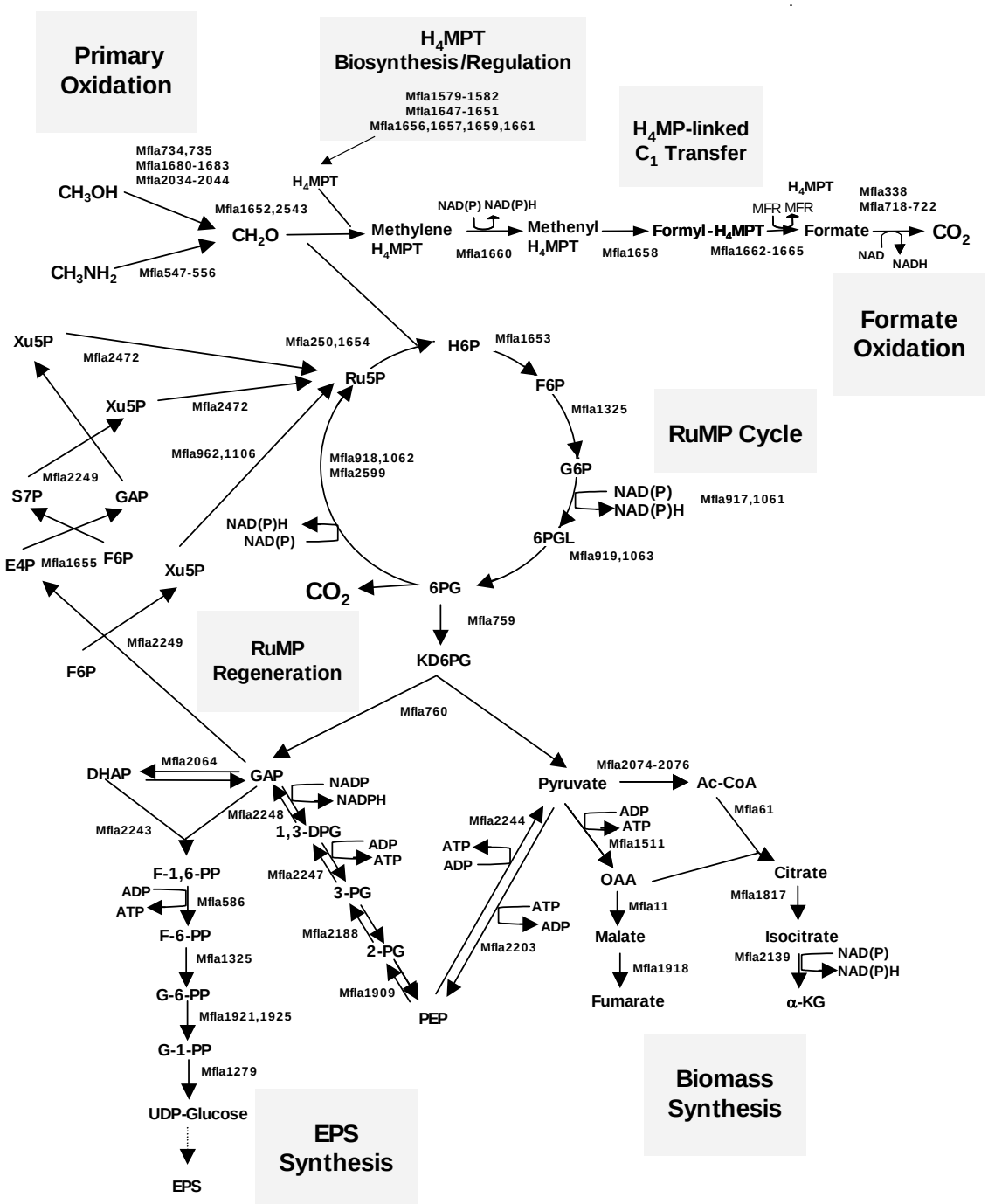
Primary Oxidation
H4MPT Biosynthesis/Regulation
Mfla1579-1582
Mfla1647-1651
Mfla1656,1657,1659,1661
H4MP-linked C1 Transfer
Mfla734,735
Mfla1680-1683
Mfla2034-2044
H4MPT
CH3OH
Mfla1652,2543
CH2O
CH3NH2
Mfla547-556
Methylene H4MPT
NAD(P) NAD(P)H
Mfla1660
Methenyl H4MPT
Mfla1658
Formyl-H4MPT
Mfla1662-1665
MFR MFR
Formate
Mfla338
Mfla718-722
CO2
NAD
NADH
Formate Oxidation
Xu5P
Mfla2472
Mfla250,1654
H6P
Mfla1653
Ru5P
F6P
Mfla1325
RuMP Cycle
Xu5P
Mfla2472
Mfla962,1106
Mfla918,1062
Mfla2599
G6P
Mfla2249
S7P
GAP
NAD(P)
NAD(P)H
Mfla917,1061
NAD(P)H
NAD(P)
6PGL
Mfla919,1063
E4P
Mfla1655
F6P
Xu5P
CO2
6PG
Mfla759
Mfla2249
RuMP Regeneration
F6P
KD6PG
Mfla760
Mfla2064
DHAP
GAP
NADP
NADPH
Mfla2074-2076
Pyruvate
Ac-CoA
Mfla2248
Mfla2243
1,3-DPG
ADP
ATP
Mfla2244
ATP
ADP
ADP
ATP
Mfla1511
Mfla61
F-1,6-PP
Mfla2247
3-PG
OAA
Citrate
ADP
ATP
Mfla586
Mfla1817
ATP
ADP
Mfla11
F-6-PP
Mfla2188
Malate
Isocitrate
Mfla1325
2-PG
Mfla2203
Mfla2139
NAD(P)
NAD(P)H
G-6-PP
Mfla1909
Mfla1918
Fumarate
α-KG
PEP
Mfla1921,1925
G-1-PP
Mfla1279
Biomass Synthesis
UDP-Glucose
EPS Synthesis
EPS

Figure 1

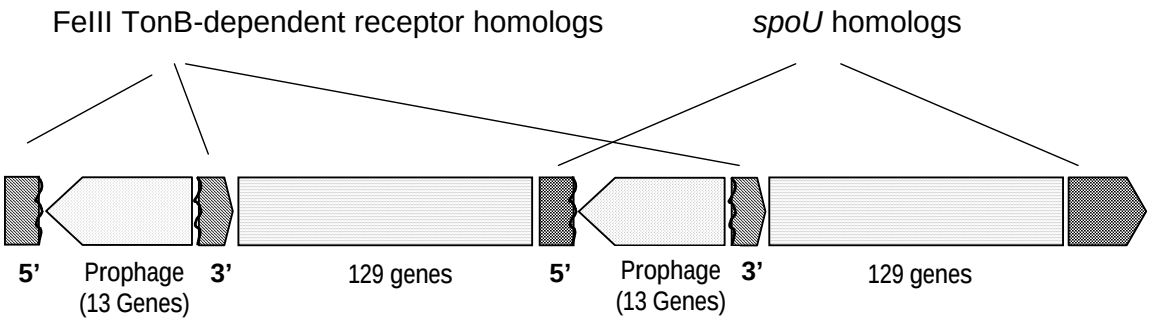
FeIII TonB-dependent receptor homologs
spoU homologs
5'
Prophage
(13 Genes)
3'
129 genes
5'
Prophage
(13 Genes)
3'
129 genes

Figure 2